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PATHOPHYSIOLOGY OF RELAPSING FEVER: INTERACTION OF BORRELIA SPI--ETC(U)
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Pathophysiology of Relapsing Fever: Interaction of Borrelia Spirochetes with Blood Mononuclear Leukocytes Causes Production of Leukocytic Pyrogen and Tissue Thromboplastin

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Abstract. Relapsing fever caused by Borrelia spirochetes is characterized by episodes of spirochetemia, fever, and disseminated intravascular coagulation (DIC). We examined the ability of Borrelia hermsii, which does not contain endotoxin, to induce production of leukocytic pyrogen and thromboplastin from human blood leukocytes in vitro. Cultures of B. hermsii were washed with pyrogen-free saline Mononuclear cells (MNC) were separated from blood by Ficoll-Hypaque gradient sedimentation and incubated with 2-5 spirochetes per MNC in 10% human serum. Supernatants from $5x10^7$ cells were assayed in rabbits for fever production. Intact MNC were simultaneously assayed for thromboplastin activity with a modified one-stage prothrombin time employing normal human plasma and plasma deficient in individual coagulation factors. Supernatants of the MNC-spirochete mixtures produced mean increases in temperature of 0.80-1.35°C, which were significantly higher than supernatants of MNC, 0.13°C, or spirochetes alone, 0.10°C ($p<0.05$). MNC-spirochete mixtures possessed 7 to 15 times the thromboplastin activity of MNC suspensions. Promotion or inhibition of phagocytosis by adding respectively immune rabbit serum or cytochalasin B did not alter production of leukocytic pyrogen or thromboplastin. In contrast, cycloheximide (10µg/ml), an inhibitor of protein synthesis, completely suppressed both pyrogen and thromboplastin production. These results suggest that fever and DIC in Borrelia infections result from an interaction between spirochetes and MNC that is independent of opsonization and phagocytosis and requires do novo synthesis of leukocytic pyrogen and thromboplastin by MNC. The Jarisch-Herxheimer reaction, which occurs after antibiotic treatment and can be fatal, may be mediated by this same mechanism and could be susceptible to inhibition.

Introduction

Relapsing fever is an acute febrile illness caused by blood spirochetes of Borrelia species. Patients with relapsing fever characteristically have high fever and abnormalities of blood coagulation, including a prolonged partial thromboplastin time and thrombocytopenia, which often give rise to a petechial skin rash (1,2,). Although the spirochetes are located predominantly free in the plasma space, the organisms have been observed also within blood phagocytic cells (3). After antibiotic therapy of relapsing fever, blood spirochetes are rapidly cleared from plasma while the rate of phogocytosis of spirochetes increases (3) and most patients undergo a Jarisch-Herxheimer-like reaction that results in a further elevation of body temperature and worsening of the abnormalities of blood coagulation (1,4). This association of enhanced phagocytosis with these pathophysiological events and the known presence of leukocytic pyrogen and tissue thromboplastin within blood leukocytes (5-8) suggested that an interaction between blood spirochetes and blood leukocytes could be responsible, in part, for the pathogenesis of relapsing fever.

The following studies seek to elucidate the mechanism of fever production and the coagulation disorder in relapsing fever. The interaction of a laboratory strain of Borrelia hermsii with human peripheral blood leukocytes under controlled in vitro conditions permitted us to define some of the conditions and metabolic requirements for phagocytosis and the generation of leukocytic pyrogen and tissue thromboplastin by the blood leukocytes.

Methods

Microorganisms. A laboratory strain of Borrelia hermsii was obtained courtesy of Dr. R. C. Johnson. It is an avirulent strain that has been serially passaged in Kelly's medium (9). Approximately 10^6 organisms were inoculated into 15 ml of Kelly's medium and incubated at 35°C for 7 days or until turbid. Viability and purity of cultures were assessed by phase contrast microscopy. Organisms were counted by direct counting in a hemacytometer through a phase contrast microscope.

Human blood leukocytes. Blood leukocytes were isolated from heparinized (20 units/ml blood) venous blood obtained from healthy volunteers using Ficoll-hypaque and dextran gradient sedimentation. After contaminating erythrocytes were removed by hypotonic lysis the cells were suspended in RPMI.

Opsonins. All leukocyte-spirochete incubations were carried out in the presence of protein that was either 10% serum or 0.5% bovine serum albumin. Some experiments contained fresh autologous serum as a source of complement. Serum was heat-inactivated at 56°C for 30 min. Immune rabbit serum was obtained from rabbits that had received intravenous injections of 10^7 formalinized Borrelia hermsii daily for 7 days followed by another injection 7 days later. The titer of anti-Borrelia antibody was measured by immobilization, agglutination, and bactericidal effects on cultures of B. hermsii (Spagnuolo and Butler, in preparation).

Assay of leukocytic pyrogen. Supernatants that produced fever in rabbits ($\geq$0.5°C rise in 2h) were examined further for the known properties of leukocytic pyrogen of heat-lability (11) by heating to 100°C for 15 min and of the requirements for de novo protein synthesis for its production by adding

cycloheximide (final concentration 10µg/ml) to the leukocyte cultures at the beginning of incubation (12).

Inhibitors of phagocytosis. Cytochalasin B was added to leukocyte cultures in final concentrations of 1, 5, and 10 µg/ml before the spirochetes were added in order to inhibit phagocytosis (13). Additionally, phenylbutazone, an inhibitor of mononuclear cell phagocytisis (14), was added to leukocyte cultures in a final concentration of 2mg/ml.

Coagulation studies. After 18 hours of incubation in RPMI, the leukocytes cultures were centrifuged at 5,000 x g for 60 min to sediment both leukocytes and any residual spirochetes. Both the sediment and supernatant were examined for the presence of tissue thromboplastin with a modified one-stage partial thromboplastin time employing human plasma deficient in individual clotting factors.

Results

Production of pyrogen by blood leukocytes after exposure to Borrelia spirochetes. Cell type. To determine whether blood leukocytes could be stimulated by spirochetes to produce a pyrogenic material, blood leukocytes were exposed to spirochetes for 30 min on a cell rotator and then after the leukocytes were washed with RPMI and incubated in RPMI for 16 hours, the supernatants were assayed for leukocyte pyrogen in rabbits. Supernatants of spirochetes alone and leukocytes without added spirochetes produced insignificant temperature rises in rabbits, but supernatants from the leukocyte-spirochete mixtures consistently produced temperature increases (Figure 1).

To determine whether the type of leukocyte producing the pyrogen was predominantly polymorphonuclear or mononuclear, these cell types were separated on Ficoll-Hypaque gradients and examined for pyrogen production after exposure to spirochetes. As shown in Figure 1, only the mononuclear leukocyte fraction produced significant temperature increases.

Evidence that the pyrogenic material induced by Borreliae is leukocytic pyrogen. Previous studies of pyrogens produced by leukocytes stimulated by endotoxin or Staph epidermidis have shown that leukocytic pyrogen is a heat-labile protein and requires de novo protein synthesis in stimulated leukocytes for its production (11,12). Supernatants from leukocyte-Borrelia mixtures that were pyrogenic were heated to 100°C for 15 min, and the pyrogenic activity was abolished. When we added the inhibitor of protein synthesis, cycloheximide, to the leukocyte cultures in a final concentration of 10 µg/ml at the same time Borreliae were added, no pyrogenic activity appeared in the supernatants (Figure 2). These properties of heat-lability and dependence of production on active protein synthesis suggested that this pyrogenic substance was leukocytic pyrogen.

Effects of protein opsonins on the production of pyrogen by Borrelia-stimulated leukocytes. Because the presence of complement and anti-Borrelial antibodies enhanced the rate of phagocytosis of spirochetes in mononuclear cell monolayers, we examined whether the protein that was present during exposure of leukocytes to spirochetes would influence pyrogen production. We compared 0.5% bovine serum albumin, autologous human serum (both fresh and heat-inactivated). All of the proteins supported production of pyrogen equally well. These results indicated that the rate of phagocytosis of Borreliae has no influence on the rate of pyrogen production by leukocytes or that this assay of pyrogen production is incapable of detecting quantitative differences in the rate of pyrogen production.

Effects of the inhibitors cytochalasin B and phenylbutazone on pyrogen production. To evaluate more directly whether phagocytosis of Borreliae by mononuclear leukocytes is a pre-requisite for pyrogen production, we employed inhibitors of phagocytosis. Both cytochalasin B 5 μg/ml and phenylbutazone 2 mg/ml have been previously shown to inhibit phagocytosis of bacteria by leukocytes without affecting viability of the leukocytes (13,14). Cytochalasin B in concentrations of 1 μg/ml and 5 μg/ml did not inhibit pyrogen production in Borrelia-stimulated mononuclear leukocytes. Only in a concentration of 10 μg/ml, which is toxic to cells, did cytochalasin B diminish the pyrogenic effect of the supernatants. At 5 μg/ml cytochalasin B did not measurably reduce pyrogen production, whether present only during initial exposure of leukocytes and Borreliae or whether re-added after the washing of the cells to be present during the entire period of incubation. Phenylbutazone also did not prevent production of pyrogen by Borrelia-stimulated mononuclear leukocytes. Because phenylbutazone has an anti-pyretic effect, as well as being an inhibitor of phagocytosis, experiments could not be performed to re-add phenylbutazone to the final incubation mixture.

Production of thromboplastin by leukocytes. Mononuclear cells, but not PMN, after exposure to spirochetes produced 7-15 times the quantity of thromboplastin that unstimulated MNC produced. Like the production of leukocytic pyrogen, the production of thromboplastin was inhibited by cycloheximide but unaffected by the absence of opsonic proteins or by the addition of cytochalasin B and phenylbutazone.

Discussion

These *in vitro* studies demonstrated that an interaction between *Borrelia* spirochetes and blood mononuclear leukocytes results in the production of leukocytic pyrogen and tissue thromboplastin by the leukocytes. Although the interaction resulted also in phagocytosis of spirochetes by the leukocytes, studies with the inhibitors of phagocytosis, cytotochalasin B and phenyl butazone, suggested that phagocytosis was not required for the stimulation of production of leukocytic pyrogen and tissue thromboplastin by the leukocytes. Thus, the stimulation of mononuclear leukocytes by *Borreliae* to produce leukocytic pyrogen and tissue thromboplastin may require only cell surface contact, without phagocytosis, between leukocytes and spirochetes in a manner similar to the stimulation of leukocytes to superoxide production by complement and immunoglobulin (13).

As in other bacterial infections characterized by fever, bacteremia, and intravascular coagulation (16), a role for blood leukocytes in producing mediators of inflammation has been suggested in relapsing fever (3,17). Blood leukocytes are known to be important sources of both leukocytic pyrogen and tissue thromboplastin, which are believed to be major mediators of fever and abnormalities of blood coagulation in infective fevers of man (5-8). Although *Borreliae* interact with and are phagocytosed by polymorphonuclear leukocytes (3), as well as by mononuclear leukocytes, our results showed that only the mononuclear leukocytes are stimulated to produce leukocytic pyorgen and tissue thromboplastin. These results are in accord with the findings of Hanson et al (18) that rabbit polymorphonuclear leukocytes do not produce leukocytic pyrogen after exposure to Staphylocci and the findings of Rivers et al and Prydz et al (19) that monocytes are the leukocytes with thromboplastin activity.

The stimulation of mononuclear leukocytes by Borreliae observed in this study resembles the action of lipopolysaccharide (LPS) from gram-negative bacteria, which also stimulates leukocytes to produce leukocytic pyrogen and tissue thromboplastin (6,7). Although some studies have suggested that Borreliae contain LPS , Butler et al tested Borrelia recurrentis directly for endotoxin activity and found spirochetes to be negative in the limulus test and in preparing rabbits for local Shwartzman reactions (2). Because the spirochetes were pyrogenic for rabbits, Borreliae contained a nonendotoxin pyrogen. The examination of Borrelia hermsii in the present study confirms these findings that Borreliae do not possess a biologically active endotoxin. Nevertheless, the nonendotoxin of Borrelia spirochetes, which has not been chemically characterized, may act in a similar manner to endotoxin by stimulating leukocytes to produce leukocytic pyrogen, tissue thromboplastin, and, perhaps, other mediators of inflammation.

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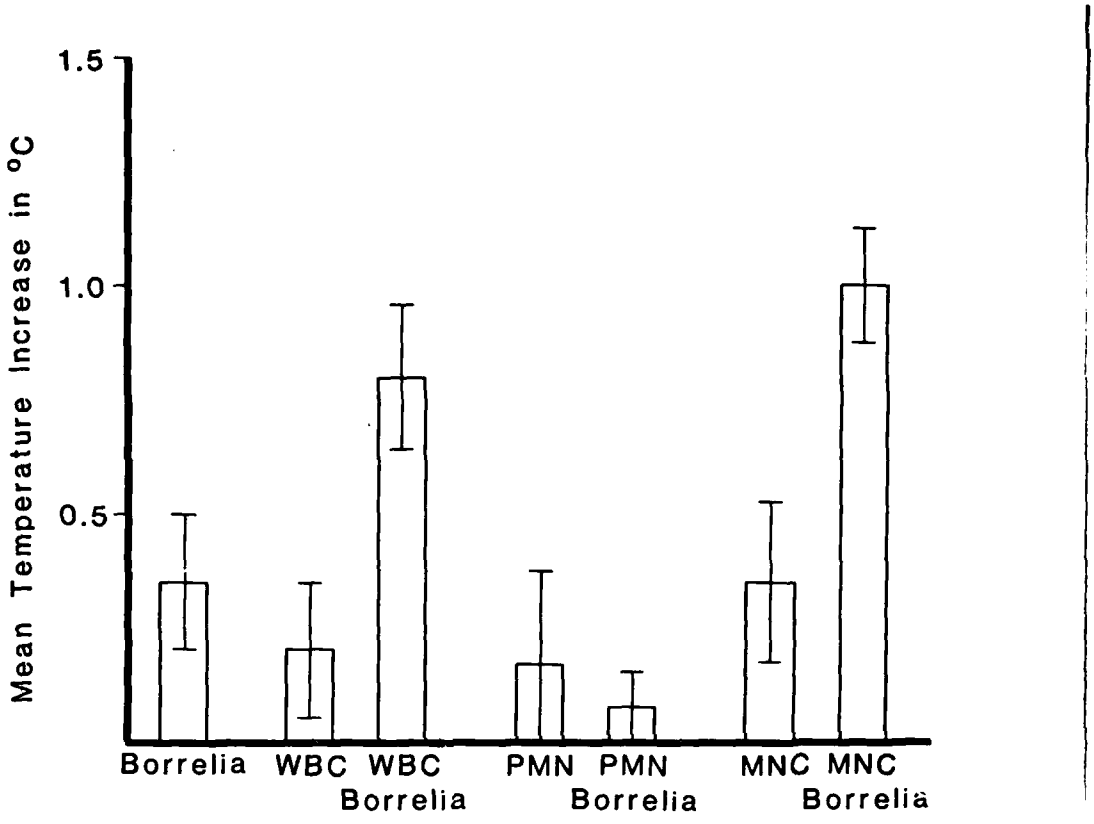


Figure 1. Action of Borrelia spirochetes to cause production of leukocytic pyrogen by human leukocytes (WBC). Polymorphonuclear leukocytes (PMN) did not produce pyrogen but mononuclear leukocytes (MNC) did produce pyrogen.

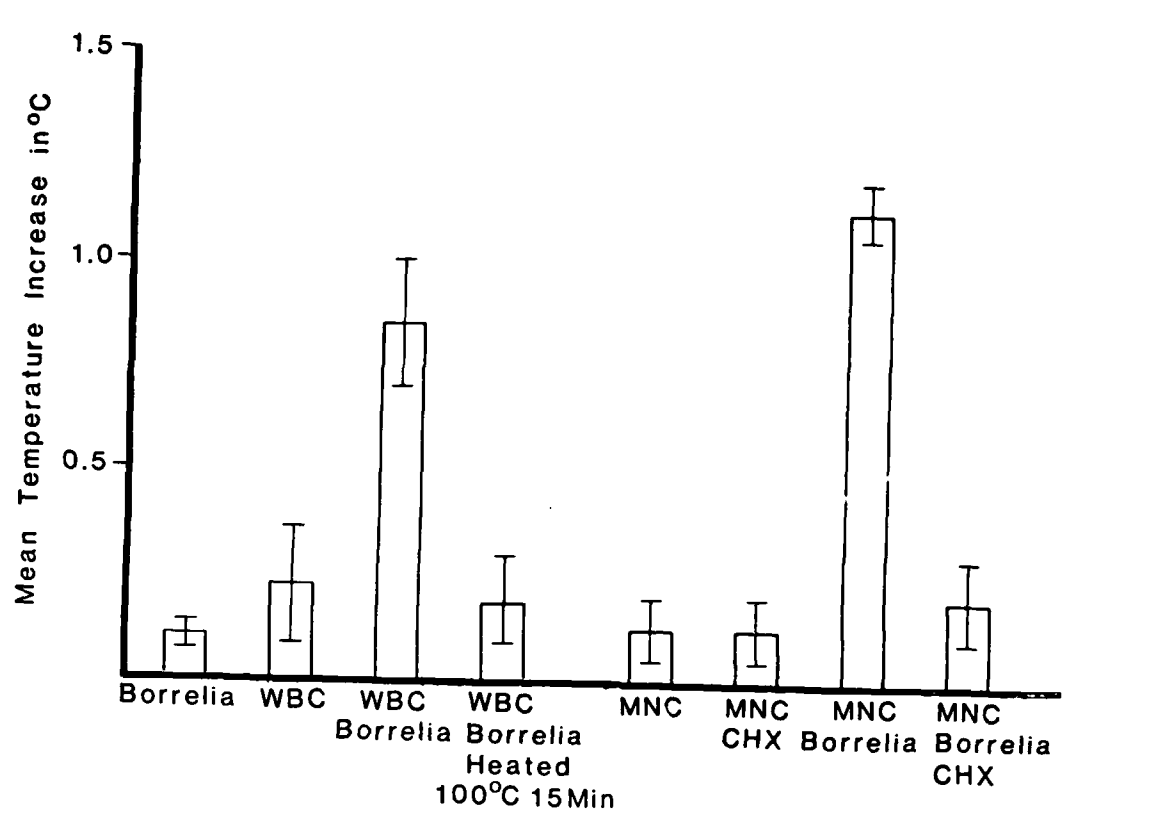
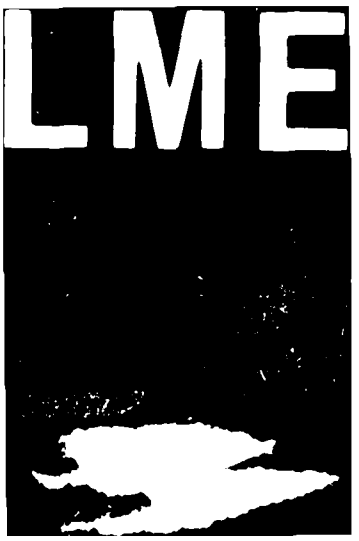


Figure 2. Leukocytic pyrogen production by Borrelia-stimulated leukocytes (WBC) is heat-labile and inhibited by cycloheximide (CHX).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